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The application of pH-sensitive fluorescent dyes in lactic acid bacteria reveals distinct extrusion systems for unmodified and conjugated dyes

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Summary

Intracellular pH in bacteria can be measured efficiently between internal pH values of 6·5 and 8·5 with the fluorescent pH indicator 2′,7′-bis-(2-carboxyethyl)-5[and-6]-carboxyfluorescein (BCECF). A new fluorescent pH probe with a lower pK_a(app) than BCECF was synthesized from fluorescein isothiocyanate and glutamate. The new probe, N-(fluorescein thio-ureanyl)-glutamate (FTUG), was much less sensitive to changes in concentrations of KCl than was BCECF. Similar to BCECF, an efflux of FTUG independent of the proton motive force, but dependent on ATP, was observed both in *Lactobacillus plantarum* and *Lactococcus lactis*. Corrections for probe efflux allowed accurate measurements of the pH_{in}. Similar intracellular pH values were determined with FTUG and BCECF, in the range where both probes can be applied, and the pH values correlated well with those estimated from the distribution of radio-labelled benzoic acid. Since FITC can easily be coupled to substrates containing an amino group, it is possible to develop other FITC derivatives as well. The mechanisms of probe excretion and the nature of the excreted product(s) were studied in further detail for BCECF and FTUG. BCECF was excreted from wild-type *L. lactis* in an unmodified form as was determined by chromatographic and mass spectrometry analysis. In the case of FTUG, the excreted product was a conjugated derivative. Unmodified FTUG was not excreted, although it was present in cellular extracts from *L. lactis*. Exit of BCECF was completely inhibited in a BCECF efflux mutant (Bef) of *L. lactis*, whereas FTUG-conjugate efflux in this mutant was similar to the wild-type. Addition of indomethacin, a known inhibitor of BCECF efflux in human epithelial cells, resulted in complete inhibition of BCECF efflux in wild-type *L. lactis*, whereas FTUG-conjugate exit was only slightly affected. The results of the mutant and inhibitor studies suggest that FTUG-conjugate and BCECF efflux in *L. lactis* are mediated by different ATP-driven extrusion systems for organic anions.

Keywords: BCECF, fluorescent pH indicators, dye conjugates, extrusion, efflux

Abbreviations: BCECF, 2′,7′-bis-(2-carboxyethyl)-5[and-6]-carboxyfluorescein; Bef , BCECF efflux negative mutant of *Lactococcus lactis* ML3; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; CFDA, 5-(and-6)-carboxyfluorescein diacetate; CHES, 2-(N-cyclohexylamino)-ethanesulphonic acid; CKC, 30 mM citric acid plus 30 mM K_2HPO_4 plus 30 mM CHES adjusted to pH 5·0–7·0 with KOH; C1-NERF,5-(and-6)-carboxy-2-chloro-3′-hydroxy-1,2,3,4-tetrahydropropyridino, [5,6-i]spiro[isobenzofuran-1(3H),9′-(9H)xanthen]-3-one; C-SNARF-1, carboxy-seminaphthorhodafluor-1; DM-NERF, 5-(and-6)-carboxy-2′,7′-dimethyl-3′-hydroxy-6′-N-ethylaminospiro[isobenzofuran-1(3H),9′-(9H)xanthen]-3-one; FITC, fluorescein isothiocyanate; FTUG, N-(fluorescein thio-ureanyl)-glutamate; MOAT, multispecific organic anion transporter; MRP, multidrug resistance-associated protein; PI, propidiumiodide.

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Introduction

The cytoplasmic pH of bacterial and eukaryotic cells determines to a large extent their physiological activity [1–3]. pH-sensitive fluorescent dyes have been applied for measuring intracellular pH (pH_{in}) in bacteria and eukaryotic cells [4]. Conventional methods to measure pH_{in} are based upon the distribution of radioactively labelled weak organic acids and bases over the cytoplasmic membrane [5, 6]. Hence, equilibration of the radioactive probes across the cytoplasmic membrane and subsequent separation of the cells from the medium by centrifugation or filtration is necessary. Another method for measuring pH_{in} uses nuclear magnetic resonance spectroscopy and is based on the pH-dependence of the chemical shift of inorganic phosphate or other compounds present in the intra- and extracellular compartments [7, 8]. Disadvantages of this method relate to the high cell densities that are required, the relatively long time intervals that are needed for each measurement, and the disturbance of the measurement by paramagnetic ions (e.g. Mn^{2+}) that are abundant in the cytoplasm of most bacteria. The use of fluorescent probes offers the advantage that pH_{in} can be measured continuously with a high time resolution and sensitivity.

Eukaryotic cells can be loaded efficiently with fluorescent indicators by adding membrane permeable acetoxymethyl ester forms of the probe to the cells [9, 10]. Intracellular hydrolysis of the probe will occur due to the action of esterases. Bacterial cells do not hydrolyse esterified fluorescent pH indicators rapidly or, alternatively, may excrete the esterified fluorescent indicator prior to trapping of the free acid in the cytoplasm [11, 12]. Another method to load bacterial cells with fluorescent indicators comprises a lowering of the external pH of a dense cell suspension for a short period of time in the presence of the probe [13]. In this way the negative charges of the probes are neutralized, allowing the acid form of the probe to enter the cytoplasm. Once inside, the probe is rapidly deprotonated and captured due to a higher pH_{in}. Other methods that have been described are covalent coupling of carboxyfluorescein succinimidylester to amino groups in proteins [14], and electroporation of the cells in the presence of the membrane impermeable fluorescent indicator pyranine [15] or dextran conjugated probes, but these methods can be detrimental for the organisms.

Most pH_{in} measurements with fluorophores have been performed with carboxyfluorescein or its derivatives. Especially 2′,7′-bis-(2-carboxyethyl)-5[and 6]-carboxyfluorescein (BCECF) is frequently used as a fluorescent pH indicator. In bacteria loaded with BCECF, an apparent efflux of the probe is observed due to the presence of excretion system(s) with specificity for BCECF [16]. When the rate kinetics of probe efflux are relatively simple, e.g. pseudo-first order, a correction of the fluorescence signal for probe efflux can be made [13].

In general the pH sensitivity of fluorophores is limited to the range pK_a(app) ± 1. Since BCECF has a pK_a (app) of 7·5, this indicator is not suitable for determination of the cytoplasmic

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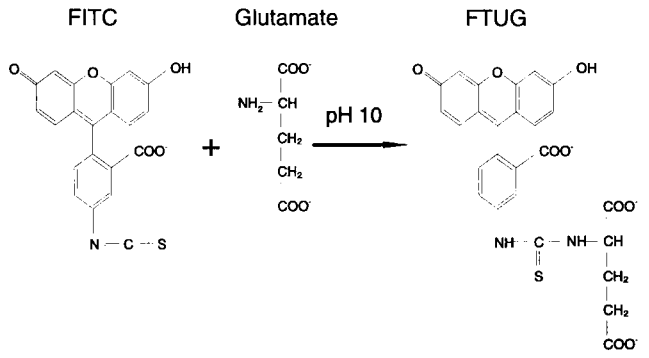

Figure 1. Reaction scheme for the synthesis of FTUG from fluorescein isothiocyanate plus glutamate.

pH below values of 6·5, which is often encountered in fermentative bacteria. Several new fluorescent indicators with a lower pK_a have been developed and applied in eukaryotic cells, e.g. 5-(and-6)-carboxy-2′,7′-dimethyl-3′-hydroxy-6′-N-ethylaminospiro[isobenzofuran-1(3H),9′-(9H)xanthen]-3-one, (DM-NERF) 5-(and-6)-carboxy-2-chloro-3′-hydroxy-1,2,3,4-tetrahydropropyridino[5,6-i]spiro[isobenzofuran-1(3H),9′-(9H) xanthen]-3-one (C1-NERF), 5-(and-6)-carboxyfluorescein diacetate (CFDA), lissamine-rhodamine B sulphonyl chloride, and carboxy-seminaphthorhodafluor-1 (C-SNARF-1) [17–19]. Unfortunately, these fluorescent indicators were not suitable for application in bacteria, due to their high rate of passive leakage from the cells (unpublished results). To expand the application of fluorescent pH indicators to more acidic pH_{in} values, a fluorescein isothiocyanate (FITC) derivative, that is well retained by bacterial cells, has been synthesized. FITC has been coupled to glutamate, yielding a new pH-sensitive fluorescent dye N-(fluorescein thio-unreanyl)-glutamate (FTUG), which has a pK_a(app) of 6·9. The new pH-sensitive dye was used to determine the pH_{in} of *Lactococcus lactis* and *Lactobacillus plantarum* over a wide pH range, and the results were compared with values obtained by the distribution of ^{14}C-benzoic acid and BCECF fluorescence.

The kinetics of probe efflux was studied for FTUG and BCECF in *Lactobacillus plantarum*, *Lactococcus lactis*, and a mutant of *Lactococcus lactis*, deficient in BCECF efflux (Bef). The results indicate that probe efflux in bacteria has features in common with BCECF efflux in human epithelial cells as well as conjugated drug transport in mammalian cells such as hepatocytes [20–22]. These putative anionic drug excretion systems are different from the previously described extrusion systems for cationic drugs in *L. lactis* [11, 23–25].

Results

Loading of bacterial cells with fluorescent dyes

The procedure to load bacterial cells with fluorescent pH indicators consists of treating a dense cell suspension with acid in the presence of the probe [13]. This procedure was successfully applied in a number of lactic acid bacteria, including *Lactococcus lactis*, *Listeria inocua*, *Leuconostoc oenos*, *Lactobacillus helveticus*, *Lactobacillus sanfransisco* and *Lactobacillus plantarum*. The efficiency and reliability of the procedure was tested in detail for *L. plantarum* and

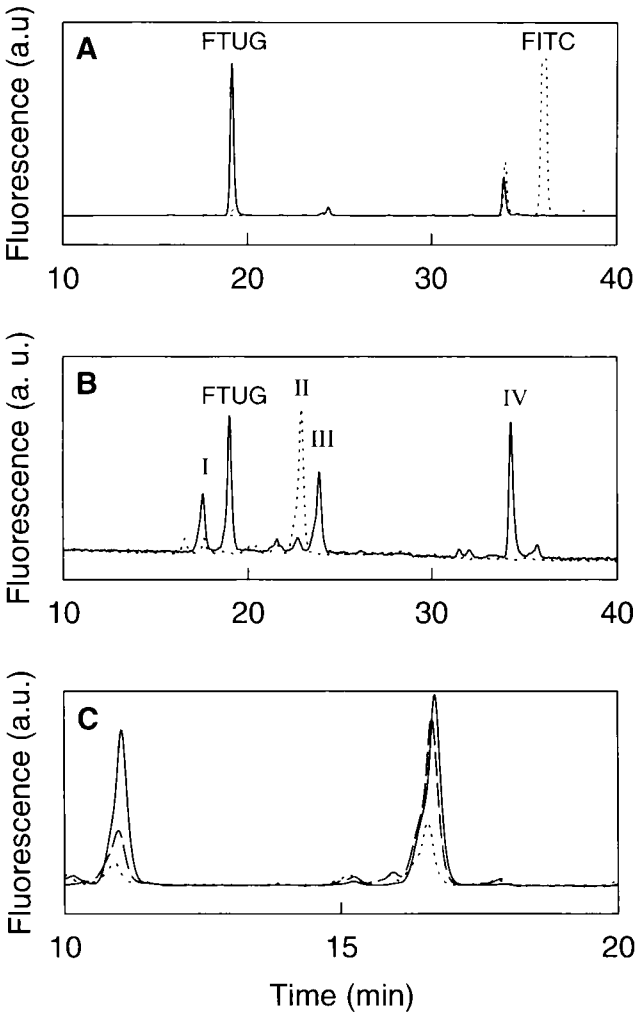

Figure 2. HPLC elution profiles of FITC, FTUG, and BCECF. **A**: Standard solutions of 5 μM FITC (dotted line) and 5 μM FTUG (straight line); **B**: intracellular FTUG (straight line) and FTUG extruded from the cytoplasm of *L. lactis* (dotted line); **C**: 5 μM standard solution of BCECF (straight line); intracellular BCECF (dashed line), and BCECF that was extruded from the cytoplasm of *L. lactis* (dotted line). *L. lactis* cells were loaded with dyes as described in materials and methods. Efflux was initiated by the addition of 10 mM glucose ($t = 0$). After 12 min of energization the cells (64 mg of protein/ml) were centrifuged and the supernatants and cellular extracts were analysed by HPLC.

L. lactis. Putative lysis of the cells was measured by using the membrane impermeant DNA stain propidiumiodide (PI), which becomes fluorescent upon intercalating with DNA following entry into the cells. For *L. plantarum* and *L. lactis* less than 3% lysis was observed in several independent experiments. Flow cytometry was used to monitor the loading of individual cells, and to show, by means of PI fluorescence, whether lysis had occurred. In the cases of *L. plantarum* and *L. lactis* approximately 1·5% of the cell population did not contain any fluorescent indicator, 95·5% was loaded with BCECF, and 3% showed PI fluorescence (data not shown). When these cells were kept on ice in the absence of glucose the leakage of BCECF or FTUG was insignificant for periods up to 8 h. The results show that the acid shock procedure can be

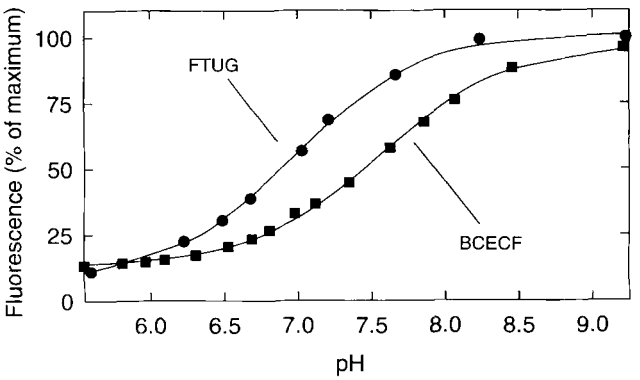

Figure 3. Titration curves of cytoplasmic BCECF and FTUG. *L. lactis* cells were loaded with BCECF (squares) or FTUG (circles) as described in materials and methods. The cells were equilibrated in CKC buffer, pH 5·0, supplemented with 1 μM valinomycin, 1 μM nigericin, plus 70 μM CCCP, and thermostated at 30 °C. The final protein concentration was 22 μg/ml. Microlitre aliquots of 5 M NaOH were added to adjust the pH, and the pH was measured simultaneously with changes in fluorescence.

used for efficient loading of bacterial cells with weakly acidic fluorescent dyes without much effect on the cell integrity.

Development of N-(fluorescein thio-unreanyl)-glutamate (FTUG)

To expand the application of fluorescent pH probes to more acidic pH_{in} values, a new pH-sensitive fluorescein isothiocyanate (FITC) derivative was synthesized. The new probe was generated by coupling of FITC to glutamate; a thio-urea bond is formed between the thiocyanate group of FITC and the amino group of glutamate (Figure 1). A 40-fold excess of the amino group containing substrate (glutamate) was added to remove the highly reactive FITC completely. The remaining products in the reaction mixture are glutamate and its derivate with FITC, that was termed N-(fluorescein thio-unreanyl)-glutamate (FTUG). In comparison to FITC, FTUG has two extra negatively charged carboxylic groups, which should minimize passive diffusion of FTUG across the cytoplasmic membrane. The synthesis reaction was followed by HPLC analysis using fluorescence detection. A good separation of FTUG (retention time 19 min), and FITC (retention time 37 min) was obtained (Figure 2A). The reaction was complete (> 99% of the FITC had reacted to a product with a retention time of 19 min) after 2 h of incubation at 4 °C in the dark. The chemical nature of FTUG was confirmed by mass spectrometry, demonstrating the conversion to a product with a molecular mass of 536 g/mol.

Characteristics of FTUG and BCECF

The fluorescence of FTUG was measured as a function of pH and compared with BCECF in dye-loaded cells of *L. plantarum* (Figure 3) and *L. lactis* (not shown) to determine the pH range in which FTUG can be used to monitor the changes in cytoplasmic pH. The ionophores valinomycin and nigericin, plus the protonophore CCCP, were added to these cells to maintain an equal internal and external pH. The titration curves show a lower pK_a(app) for FTUG (6·9) than for BCECF (7·5) both in *L. plantarum* (Figure 3) and *L. lactis* (data not shown). Some variation in pK_a(app) values was observed, especially between different batches of BCECF (7·3–7·7) and to a lesser extent also for FTUG (6·9–7·0). The variation within one bath of probe was limited to approximately 0·05 pH unit. Since a pH-sensitive fluorescent dye might also respond to cations other than protons, the influence of sodium and potassium ions on the fluorescence quantum yield of BCECF and FTUG was tested next. To investigate the effect of ionic strength on the pK_a(app) of BCECF and FTUG, titration curves were made in the presence and absence of 1 M KCl, a physiological concentration in the cytoplasm of lactic acid bacteria [26, 27]. The pK_a(app) of FTUG was lowered from 6·9 to 6·7, whereas that of BCECF was lowered from 7·5 to 6·7.

Intracellular pH measurements

Upon addition of glucose to dye-loaded cells the fluorescence increases, which corresponds with an increase in pH_{in}. After this initial increase the fluorescence decreases slowly in time

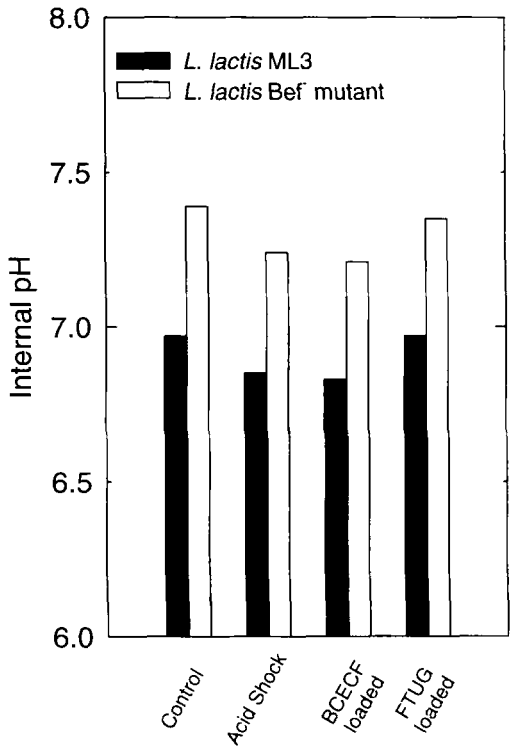

Figure 4. Cytoplasmic pH of *L. lactis* ML3 and *L. lactis* ML3 Bef⁻, with and without BCECF or FTUG. The cells were loaded with BCECF or FTUG as described in materials and methods. The loaded cells were diluted into 50 mM potassium phosphate, pH 5·9, thermostated at 30 °C, containing 16 μM ^{14}C-benzoic acid (50 mCi/mmol), and 10 mM glucose. The final protein concentrations were 0·52 and 1·03 mg/ml for *L. lactis* ML3 (closed bars) and *L. lactis* ML3 Bef⁻ (open bars), respectively. After 10 min of energization the cells were separated from the medium by silicone oil centrifugation. The maximal error in pH_{in} between independent assays comprised 0·15 pH unit (triplicate measurements).

Table 1. The intracellular pH of *L. lactis* and *L. plantarum* determined by the distribution of ^{14}C-benzoic acid and the fluorescence of BCECF and FTUG.

	Internal pH		
External pH	^{14}C-benzoic acid distribution	FTUG fluorescence	BCECF fluorescence
L. lactis			
5·5	7·02	7·00	7·02
6·0	7·11	7·10	7·10
6·5	7·36	7·53	7·30
7·0	7·68	7·81	7·67
L. plantarum			
5·0	—	6·40	n.d.
6·0	—	7·05	7·13
7·0	—	7·33	7·35

The internal pH measurements were performed in CKC buffer of the desired pH at a final protein concentration of 0·78 mg/ml (distribution of benzoic acid) and 8 μg/ml (fluorescence measurements). n.d. = not detected (the fluorescence quantum yield was too low); — = not done.

as a result of probe efflux (see below) and falls back to the initial values upon dissipation of the pH gradient by the ionophore nigericin. To test whether dye-loading affects the pH_{in} of *L. lactis* ML3, the pH_{in} of unloaded cells was compared with that of BCECF- and FTUG-loaded cells by measuring the distribution of ^{14}C-labelled benzoic acid across the membrane. Loading of *L. lactis* ML3 with FTUG or BCECF had no significant effect on the pH_{in} values (Figure 4; closed bars). Moreover, a good correlation (especially at lower pH values) was observed of the absolute pH values obtained from the distribution of ^{14}C-benzoic acid and from the fluorescence of BCECF and FTUG over the pH_{ex} range 5·0–7·0 (Table 1). These results demonstrate that FTUG can be used for the measurement of pH_{in} values that are approximately 0·5 pH unit lower than the pH values that can be measured with BCECF, and that FTUG is less sensitive to changes in potassium concentration than is BCECF.

Efflux of FTUG and BCECF

The kinetics and energetics of the efflux of FTUG and BCECF in *L. plantarum* were studied in the presence and absence of glucose. In contrast to energy-depleted cells, glycolysing cells excreted both probes on a 10 min time scale, indicating that excretion is a metabolic energy-requiring process. Manipulation of the proton motive force by the addition of valinomycin and/or nigericin had no effect on the rate of BCECF and FTUG efflux in *L. plantarum* (Figure 5). This suggests that excretion of FTUG and BCECF is most likely ATP-driven in *L. plantarum* as was suggested previously for BCECF efflux in *L. lactis* [16].

To investigate whether BCECF and FTUG excretion are mediated via the same system, the efflux of both dyes was further characterized in *L. lactis* ML3, since an isogenic BCECF efflux mutant (Bef$^-$ mutant) was available for this strain [16]. Although the Bef$^-$ mutant grows equally well as the parent ML3 strain [16], it was observed that the mutant maintains the pH_{in} at a value approximately 0·4 pH unit higher than the wild-type (Figure 4). Since the defect of this mutant in BCECF efflux was also observed under conditions in which $pH_{in} = pH_{out}$, it is unlikely that the reduced efflux rate is a direct consequence of the higher pH_{in}. The efflux of FTUG was not affected in the Bef$^-$ mutant (Figure 6), whereas BCECF efflux was slowed down more than five-fold. This suggests that FTUG is excreted by a separate efflux system or, alternatively, that the apparent affinity constant for BCECF is increased in the Bef$^-$ mutant. Excretion of BCECF occurred at a higher rate than FTUG in *L. lactis*, whereas the opposite was observed in *L. plantarum* (Figures 5 and 6).

Chemical nature of intracellular FTUG and BCECF

To establish whether the fluorescent probes are modified, the chromatographic behaviour of excreted products was compared with standard solutions of BCECF and FTUG. These experiments showed that the excreted product from BCECF-loaded *L. lactis* (dotted line) eluted at retention times similar to authentic BCECF (straight line). BCECF from cytoplasmic

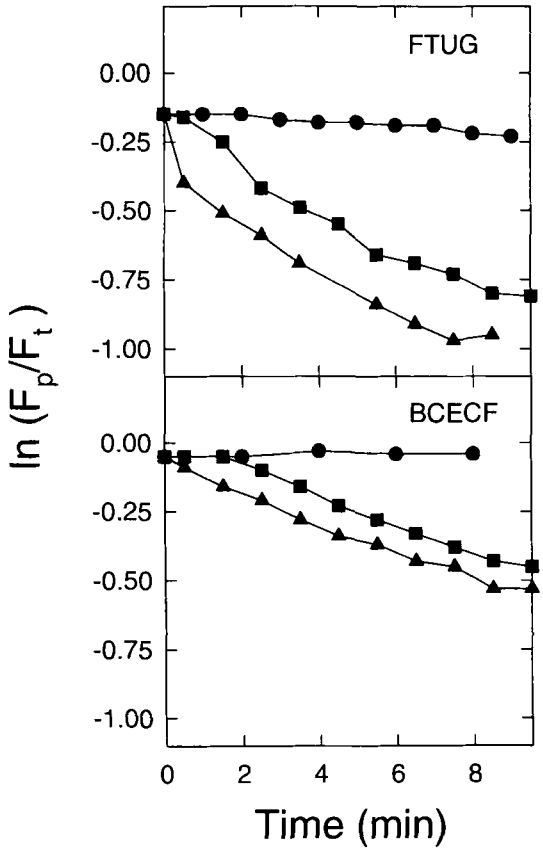

Figure 5. Efflux of BCECF and FTUG from *L. plantarum*. The cells were loaded with BCECF or FTUG as described in materials and methods. The loaded cells were diluted to a final protein concentration of 2·0 mg/ml into 50 mM potassium phosphate, pH 6·5, thermostated at 30°C. At various times 200 μl aliquots were taken and rapidly centrifuged to separate internal and external fluorescent indicators. F_p is the fluorescence of the pellet fraction after disruption and centrifugation to remove debris, F_t is the total fluorescence (pellet plus supernatant). The efflux was followed in the absence of an energy source (circles), or in the presence of 10 mM glucose without (squares) and with 1 μM valinomycin plus nigericin (triangles). The standard errors of the mean were less than reflected by the size of the symbols (i.e. when data were collected from a single batch of cells).

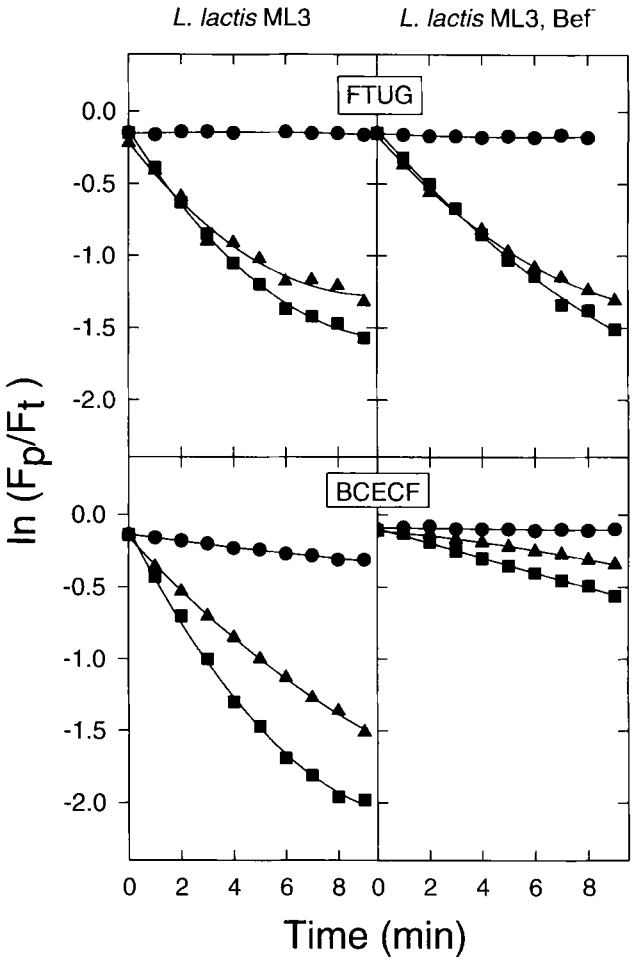

Figure 6. Efflux of BCECF and FTUG from *L. lactis* ML3 and Bef mutant. The cells were loaded with BCECF or FTUG as described in materials and methods. The loaded cells were diluted to a final protein concentration of 0·66 mg/ml and 0·70 mg/ml for *L. lactis* ML3 and *L. lactis* ML3 Bef⁻, respectively, into 50 mM potassium phosphate, pH 6·5, thermostated at 30 °C. At various times 200 µl aliquots were taken and further handled as described in the legend to Figure 5. Symbols are the same as in the legend to Figure 5.

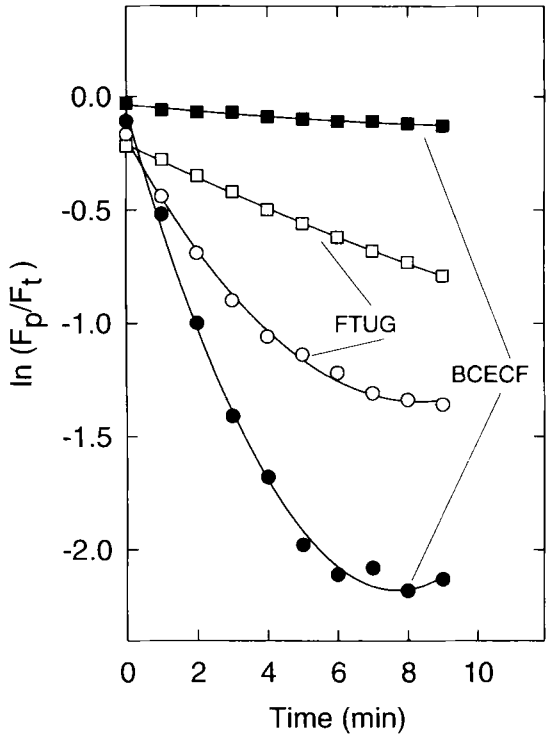

Figure 7. Effect of indomethacin on BCECF and FTUG efflux in *L. lactis* ML3. The cells were loaded with BCECF or FTUG as described in materials and methods. The loaded cells were diluted to a final protein concentration of 0·44 mg/ml into 50 mM potassium phosphate, pH 6·5, thermostated at 30 °C. At various times 200 µl aliquots were taken and further handled as described in the legend to Figure 5. The efflux of BCECF (closed symbols) and FTUG (open symbols) was followed in the presence of 10 mM glucose without (circles) and with 1 mM indomethacin (squares).

extracts of *L. lactis* (dashed line) was also found to be identical to the original probe (Figure 2C). About 70% of the internal FTUG, however, eluted at different retention times from the original probe, mostly at 17·5 min (13%; peak I), 23·5 min (20%; peak III), and 34·0 min (28%; peak IV). The product eluting at 34 min (peak IV) is a contamination that is also present in FITC and FTUG standard solutions. One fluorescent FTUG-derivative, that was present in a low concentration in cellular extracts (±3% of the total area; peak II), was preferentially excreted by *L. lactis* (Figure 2B).

Mass spectrometry analysis confirmed that excreted BCECF corresponds to a compound with a molecular weight of 520 g/mol, which is identical to the calculated M_w of BCECF. The commercially available BCECF, however, consists of two isomers that were separated by HPLC (Figure 2C). The peaks could not be assigned to either 2′,7′-bis-(2-carboxyethyl)-5-carboxyfluorescein or 2′,7′-bis-(2-carboxyethyl)-6-carboxyfluorescein, because it is not possible to obtain optically pure BCECF. The ratio between the two isomers with retention times of 17·2 and 11·5 min, respectively, decreased from 0·85 for free BCECF to 0·28 in cytoplasmic extracts of *L. lactis*, and remained the same after extrusion from the cytoplasm into the surrounding medium (Figure 2C). This indicates that the cells are more efficiently loaded with one of the isomers of BCECF, but that the efflux system does not discriminate between the two isomers.

Inhibitor studies

Indomethacin, which is known to abolish BCECF efflux in human epithelial cells [1, 9], was used to characterize the efflux of BCECF and FTUG-derivative further. Addition of 1 mM indomethacin completely inhibited BCECF excretion in *L. lactis* ML3, whereas only a 50% reduction of 'FTUG' efflux was observed under these conditions (Figure 7). Indomethacin decreased the pH_{in} of glycolysing cells by 0·25 pH unit (not shown), but this effect is unlikely to be the cause for the inhibition of probe efflux as BCECF excretion is slightly lowered and 'FTUG' excretion is not affected by changes in pH_{in} (Figures 5 and 6).

Discussion

In this paper the synthesis and characterization of N-(fluorescein thio-ureanyl)-glutamate (FTUG), a new pH-sensitive fluorescent indicator with a pK_a(app) of 6·8, is described. The probe is readily synthesized from fluorescein isothiocyanate plus glutamate, and its retention by *L. plantarum* and *L. lactis* is comparable to that of BCECF. After correction for probe efflux the fluorescence signal can be transformed to cytoplasmic pH values via a calibration curve, as was previously described for BCECF [13]. In *L. lactis* the pH_{in} values estimated from the distribution of ^{14}C-benzoic acid and the fluorescence of both BCECF and FTUG were similar. Such a good correlation between ^{14}C-benzoic acid distribution and BCECF fluorescence data was not concluded from previous experiments with *L. lactis*; the pH values obtained from BCECF fluorescence were 0·3 pH unit higher than those obtained from the ^{14}C-benzoic acid distribution [13]. It seems likely that these differences are due to variations in the physiological state of the cells, as these comparisons were made between cells that were not cultivated in an identical manner. In our experiments the internal pH values derived from the fluorescence and benzoic acid data were obtained with the same batches of cells.

The fluorescence changes of BCECF and FTUG were tested for potential artifacts, such as sensitivity to cations and variations in different batches of probes that could affect the pH_{in} determinations. A lowering of the pK_a(app) from 7·5 to 6·7 in the presence of 1 M KCl was observed for BCECF. The effects of K^+ on the pK_a(app) of FTUG were much less: the pK_a(app) decreased from 6·9 to 6·7 when KCl was added at 1 M. This makes FTUG a more reliable probe for the estimation of cytoplasmic pH values, as changes in potassium concentration are frequently associated with changes in pH_{in} [1]. Moreover, the pK_a(app) of BCECF varied considerably between different batches of probe (from 7·3 to 7·7), whereas the pK_a(app) of various batches of FTUG was quite constant (6·9–7·0). In parallel with the difference in pK_a(app), the ratio between the two isomers of BCECF also varied between different batches of BCECF (the primary eluting isomer in HPLC chromatograms (Figure 2C) constituted between 40% and 55% of the total BCECF). Both observations were confirmed by Molecular Probes Inc. The manufacturer, however, was not able to provide information about the pK_a(app) of the two different isomers. It seems likely that the variation in pK_a(app) between separate batches of BCECF is caused by a different pK_a(app) of the two isomers.

Although both FTUG and BCECF are excreted by *L. lactis*, *L. plantarum*, and other bacteria (unpublished results), this efflux does not seriously hamper the use of the probes as indicators of pH_{in}. In *L. lactis* the efflux rate of BCECF was somewhat lowered upon the addition of valinomycin plus nigericin. This is most likely due to a decrease in the activity of the extrusion system at the lower pH_{in} values. The efflux of FTUG was not affected by valinomycin plus nigericin, which is consistent with a separate efflux system for this probe (see below). In *L. plantarum*, efflux of BCECF and FTUG is initially slow, which corresponds with a slow start of glycolysis, and consequently a lag in the production of ATP. Since efflux of BCECF and FTUG is independent of a proton motive force in both *L. lactis* and *L. plantarum*, but dependent on metabolic energy, we speculate that the extrusion of both compounds is driven by ATP directly.

To investigate whether BCECF and FTUG are modified prior to excretion from the cells, HPLC chromatograms of free BCECF (or FTUG) in solution and excreted products were compared. Excreted BCECF, as well as BCECF in cytoplasmic extracts of *L. lactis*, eluted at similar times as unmodified BCECF in solution. However, the retention time of 70% of the FTUG in cytoplasmic extracts from dye-loaded *L. lactis* did not correlate with that of a standard solution, and one of these products was preferentially excreted. As already suggested by the different sensitivity towards a lowering of pH_{in} (Figure 6), this could indicate that the mechanisms of BCECF and FTUG efflux are different. Further support for this notion came from studies in the BCECF efflux mutant of *L. lactis* ML3 (Bef) [16], in which, in contrast to excretion of BCECF, the efflux of the FTUG conjugate was identical to the parent ML3 strain. In mammalian cells transport of various organic anions occurs via the multispecific organic anion transporter (MOAT), also termed multidrug resistance-associated protein (MRP) [21, 28–30]. The MOAT protein resembles P-glycoprotein in its primary sequence as well as function; both proteins are ATP-dependent export systems which are regulated by phosphokinase c, and pump a wide variety of organic compounds out of the cell against high concentration gradients [21, 22, 31]. However, P-glycoprotein excretes cationic and neutral substrates, whereas MOAT is specific for anions (cysteinyl leukotrienes, glutathione *S*-conjugates, and other amphiphilic anions). Typical substrates/inhibitors of P-glycoprotein (e.g. daunorubicin, cyclosporin A (analogues) and verapamil) do not affect the export of organic anions via cMOAT [20]. The observation that FTUG is excreted in a modified form is reminiscent of the transport of (mostly) glutathione conjugates by MOAT in mammalian cells. Unfortunately, the concentrations of the FTUG derivates were too low to determine their molecular masses by mass spectrometry, or to isolate these compounds for structure analysis. *In-vitro* conjugation of FTUG to components of cellular extracts or glutathione has failed thus far, most likely due to the absence of essential cofactor(s). Identification of the FTUG derivatives will be the topic of our future research.

In human epithelial cell lines the pharmacological profile of the inhibition of BCECF efflux was found to be unique, i.e. not resembling the inhibitors for P-glycoprotein or MOAT mediated efflux [32, 33]. A potent and specific inhibitor of BCECF efflux is indomethacin. When indomethacin was added to *L. lactis* cells excreting BCECF or FTUG, the efflux of FTUG was somewhat lower, but BCECF efflux was almost completely inhibited. Although the affinity constants for BCECF and 'FTUG' excretion and indomethacin inhibition have not been determined, this result also is consistent with the presence of two distinct extrusion systems for organic anions in *L. lactis*. The efflux of BCECF may occur via a similar system to that present in human epithelial cells, whereas FTUG-conjugate efflux might be related to the MOAT (or MRP) efflux system that is observed in various mammalian cells.

In conclusion, the characterization of the bacterial retention of a new fluorescent pH indicator has led to the identification of

an efflux activity that may represent part of a hithertho unforeseen drug detoxification mechanism in bacteria. The isolation of a FTUG efflux mutant and determination of the nature of the excreted product should form important steps forward in the further unravelling of this excretion mechanism.

Experimental procedures

Media and strains

Lactobacillus plantarum VBLLAC11-10 (typed by the American Type Culture Collection) was kindly provided by Unilever Research Laboratories. *Lactococcus lactis* subspecies *lactis* ML3 and the BCECF efflux mutants (Bef) have been described previously [16]. All organisms were grown at 30 C on a modified MRS medium [34], containing (per litre) 10 g of tryptone, 10 g of LabLemco powder (Oxoid), 5 g of yeast extract, 2 g of K_2HPO_4, 0·2 g of $MgSO_4 . 7H_2O$, 0·035 g of $MnSO_4 . H_2O$, and 1 ml of Tween 80, adjusted to pH 6·6 with HC1. The medium was supplemented with separately sterilized 0·5% (w/v) glucose.

Synthesis of N-(fluorescein thio-unreanyl)-glutamate (FTUG)

Fluorescein isothiocyanate (final concentration, 11 mM) was freshly prepared in 90 μl of 50 mM bis-Tris-Propane, pH 10·0. The reaction was initiated by adding 10 μl of 4 M K-glutamate, pH 10·0. The reagents were mixed thoroughly and kept at 4 C in the dark for 2–18 h to complete the reaction. The mixture was used directly.

Loading of bacteria with BCECF or FTUG

Cells were harvested in the logarithmic phase of growth at an OD_{660} of 0·6, washed twice with 50 mM potassium phosphate, pH 7·0, and resuspended to a final protein concentration of 10–50 mg/ml. The fluorescent indicator (BCECF or FTUG) was added to these cells to a final concentration of 1 mM. The cells were loaded by lowering the external pH to approximately 1 for 5 min by adding a small aliquot of 0·5 M HC1 [13]. Routinely, the amount of acid used was 2·5 μl of 0·5 M HC1 per 20 μl of concentrated cell suspension (50 mg/ml). Subsequently, the cells were washed four times with 50 mM potassium phosphate, pH 7·0.

Internal pH measurements

Fluorescence measurements on dye-loaded cells were performed in 30 mM citric acid, 30 mM K_2HPO_4, 30 mM 2-(N-cyclohexylamino)-ethanesulphonic acid (CHES), adjusted to pH 5·0–7·0 with KOH (CKC buffer). The final protein concentration during the measurement was 10–25 μg/ml. Excitation and emission monochromator wavelengths for BCECF were 502 and 525 nm, with slit widths of 5 and 15 nm, respectively. For FTUG excitation and emission wavelengths were 495 and 518 nm, with slit widths of 2·5 and 20 nm, respectively. The fluorescence signal was averaged over time intervals of 1 s. The cytoplasmic pH was calculated from the fluorescence data with the Henderson–Hasselbach equation:

$$\mathrm{pH} = \mathrm{p}k_a(\mathrm{app}) + \log\,(F - F_{\min}/F_{\max} - F),$$

in which $F. F_{\min}$ and $F_{\max}$ correspond to the actual fluorescence signal that is measured, the minimal fluorescence that is measured at low pH (< 5·0) in uncoupled cells (in the presence of 1 μM valinomycin, 1 μM nigericin, plus 70 μM N-carbonyl cyanide *m*-chlorophenylhydrazone (CCCP)), and the maximal fluorescence that is measured at high pH (> 10·0) in uncoupled cells.

The signal was corrected for probe efflux according to Molenaar *et al.* [13]:

$$F_{\mathrm{cor}} = (e^{kt}/q_0) . (F - (1 - q_0 . e^{-kt}) . F_0)$$

in which F_{cor} is the fluorescence signal that was corrected for probe efflux, F is the total fluorescence signal that is measured, F_0 is the fluorescence when all fluorescent indicator is located extracellularly (determined by the addition of 0·2% Triton X-100), k is the first-order rate constant for efflux (determined by plotting ln $|F - F_0|$ against time), and q_0 is the fraction of the indicator that was located internally at time zero (which is close to 1). The fluorescent techniques to determine pH_{in} were compared with the distribution of ^{14}C-benzoic acid (specific activity 50 mCi/mmol) over the cytoplasmic membrane using silicone oil centrifugation to separate the cells from the surrounding medium [35].

Efflux assays

Cells, loaded with BCECF or FTUG, were diluted into 2 ml CKC buffer of the desired pH to a final protein concentration of 0·5–2 mg/ml at 30 C. After 1 min of incubation the assays were initiated by adding glucose (final concentration 10 mM) with or without 1 mM indomethacin or 1 μM each of valinomycin plus nigericin. Aliquots of 200 μl were withdrawn and the cells were spun down within 20 s in a microfuge at 14 000 *g*. The supernatant (180 μl) was removed carefully and the fluorescence corresponding to extracellular probe was measured after a 16·7-fold dilution with bis-Trispropane, pH 10·0. The pellet was resuspended in 160 μl 0·2% Triton X-100, and the suspension was kept at room temperature for 30–60 min to release internal fluorescent dyes. Subsequently, the cell debris was spun down for 5 min in a microfuge and the fluorescence was determined as before. Alternative methods to permeabilize the cells (e.g. sonication, higher concentrations of detergent) confirmed that 0·2% Triton X-100 was sufficient to permeabilize the cells.

HPLC analysis

BCECF and FTUG solutions (50 μl) were applied to an Econosphere C_{18} reversed-phase column using fluorescence detection (excitation at 500 nm, emission at 520 nm). The probes were eluted with a gradient of 0·4% glacial acetic acid, pH 4·9, and acetonitril. The elution was initiated ($t = 0$) with 0% acetonitril, subsequently the concentration of acetonitril was increased to 15% at $t = 20$ min and 30% at $t = 30$ min; elution with 30% acetonitril was continued for 10 min, whereafter the column was equilibrated with buffer (0% acetonitril) for 15 min. Cellular extracts and excreted dyes were obtained from dye-loaded cells (see above) in 50 mM potassium phosphate, pH 6·5, at a final protein concentration of about 50 mg/ml, essentially as described in the section 'efflux assays'.

Non-energized cells (control) and cells that were actively excreting the dyes for 20 min by energization with 0·2% glucose at 30 °C were collected by centrifugation at given times. Supernatants were analysed directly, and the pellets were extracted by adding 300 μl of methanol, followed by 5 min incubation at room temperature and removal of cellular debris by centrifugation. Mass spectrometry analysis of BCECF and FTUG solutions was performed by direct flow-injection.

Flow cytometry

Cells were loaded with BCECF and FTUG as described above and, immediately after loading, 1 μg/ml of the membrane-impermeable DNA stain propidiumiodide was added to the cell suspension. To increase the sensitivity of the flow cytometric detection the cells were counterstained with the membrane-permeable DNA stain Hoechst (1 μg/ml). Flow cytometric analysis was performed approximately 3 h after loading.

Miscellaneous

Protein was determined by the method of Lowry *et al.* [36] with bovine serum albumin as a standard. Distributions of ^{14}C-benzoic acid were calculated using water-accessible cytoplasmic volumes of 2·5 and 2·9 μl/mg of protein for *Lactobacillus plantarum* and *Lactococcus lactis*, respectively [37, 38].

Chemicals and equipment

Fluorescent dyes were obtained from Molecular Probes, Eugene, OR, USA. BCECF solutions were prepared as described by Molenaar *et al.* [13] and stored at −20 °C in the dark. Radiochemicals were purchased from Amersham (Buckinghamshire, UK). All other chemicals were reagent grade and obtained from commercial sources. The fluorimeter for pH determinations was a Perkin-Elmer LS50 with computer-controlled data acquisition and storage. The flow cytometer was a Fluorescence Activated Cell Sorter II (FACS II).

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